Selective Activation of Ceruloplasmin Promoter in Ovarian Tumors: Potential Use for Gene Therapy

Christine M. Lee,1,3 Hui-Wen Lo,2 Ru-Ping Shao,2 Shao-Chun Wang,2 Weiya Xia,2 David M. Gershenson,1 and Mien-Chie Hung2

Departments of 1Gynecologic Oncology, 2Molecular and Cellular Oncology, 3Graduate School of Biomedical Sciences, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Gene therapy provides a novel treatment approach to cancer patients. Ideally, expression of therapeutic genes driven by cancer-specific promoters would only target tumors resulting in minimal toxicity to normal tissues. While there is a need of more effective and tolerable treatments for ovarian cancer patients, we aimed to identify gene promoters with high activity in ovarian tumors that can be potentially used in gene therapy to drive the expression of a therapeutic gene in tumors. To identify such promoters, a literature search was performed to reveal genes that are preferentially expressed in ovarian cancer compared with normal ovaries. We found that the ceruloplasmin promoter drove up to 30-fold higher luciferase expression in ovarian cancer cells compared with immortalized normal cells. Furthermore, deletion studies revealed an activator protein-1 (AP-1) site in the ceruloplasmin promoter to be critical for optimal ceruloplasmin promoter activity. Ceruloplasmin promoter activity was significantly activated by 1-O-tetradecanoyl phorbol-13-acetate, a c-jun activator, and conversely suppressed by SP600125, a c-jun inhibitor. Consistently, the ceruloplasmin AP-1 site was specifically recognized by c-jun both in vitro and in vivo. Immunohistochemical analyses of human ovarian cancer specimens showed a direct correlation between expression levels of c-jun and ceruloplasmin. In nude mice carrying SKOV3.ip1 xenografts, the ceruloplasmin promoter demonstrated significantly higher activity in tumors compared with normal organs. Together, these results suggest that the ceruloplasmin promoter activity is significantly enhanced in ovarian cancer and therefore may be exploited as a promising cancer-specific promoter in developing new gene therapy strategies for ovarian cancer.

INTRODUCTION

In the United States, ovarian cancer is the leading cause of cancer death of the female genital tract. In 2002, approximately 13,900 women died of the disease (1). The standard of care for patients with advanced-stage ovarian cancer since 1996 is optimal surgical debulking followed by platinum-based chemotherapy (2). Although 80% of patients who are treated with this regimen initially demonstrate a clinical response (2), the majority of patients will recur. There are major limitations of effective therapeutic options for patients with advanced ovarian cancer who do not respond to initial therapy or those with recurrent disease. Therefore, identification of effective and more tolerable treatment methods is needed to provide patients with other options in treating ovarian cancer. Delivering therapy directly to the abdominal cavity may be beneficial for patients by directing treatment to the site of the tumor.

Gene therapy is a potentially useful therapeutic tool for patients with ovarian carcinoma because it can be delivered directly to the intra-abdominal cavity where the bulk of the tumor develops (3). To develop an ovarian cancer-specific promoter (CSP), we first sought to determine the genes that are highly expressed in ovarian cancer because we reasoned that some of the genes that are preferentially expressed in ovarian cancer are transcriptionally activated. That is, the overexpression of genes in ovarian cancer may be due to strong promoter activity. We reasoned that if overexpression is due to transcriptional up-regulation, the promoters of the overexpressed genes would be more active in ovarian cancer than in normal cells, and therefore can be used to drive a therapeutic gene to target ovarian cancer cells for effective gene therapy and minimizing side effects. Based on relative expression ratios (ovarian cancer versus normal) available in the literature, we have identified several promoters that are more active in ovarian cancer cells (4–6). Among these, the ceruloplasmin promoter is more specific to ovarian cancer cells.

The ceruloplasmin serves as a cofactor in various physiological enzymatic reactions including a role in copper transport (7), maintenance of vessel tone (8–10), and antioxidant properties, which has implications in disorders including Parkinson’s and Alzheimer’s diseases (11). High levels of ceruloplasmin expression have been demonstrated in various cancers such as thyroid carcinoma (12) and melanoma (13). Dysregulation of copper transport due to ceruloplasmin expression in tumors has been studied by suppressing copper with tetrathiomolybdate in head and neck tumors in clinical trials (13).

The use of the ceruloplasmin promoter as a potential tool for gene therapy in cancer has not been previously investigated. It was originally identified and cloned with evidence that the regulatory CAAT/enhancer-binding protein elements were not responsible for tissue specificity (14). Mutations in the ceruloplasmin promoter appear not to be responsible for the dysfunction in iron modulation in patients with diseases such as hemochromatosis (15). The ceruloplasmin promoter appears to be regulated by hypoxia-responsive elements and hypoxia-inducible factor-1 (16) as well as iron deficiency anemia (16). Because the ceruloplasmin promoter is regulated by hypoxia and anemia, it may serve as a useful tool in gene therapy because these altered physiological mechanisms are commonly seen in patients with ovarian cancer.

We initiated this study to investigate the potential of the ceruloplasmin promoter in enhancing the specificity and efficacy of targeting ovarian cancer. The findings reported in this study define the ceruloplasmin gene promoter as a novel and potent CSP targeting ovarian cancer. Functional characterization of the ceruloplasmin promoter suggests that the ceruloplasmin AP-1 site is involved in the c-jun-mediated overexpression of ceruloplasmin in ovarian cancer.

MATERIALS AND METHODS

Reagents and Plasmids. Opti-MEM1 reduced serum media was purchased from Invitrogen (Carlsbad, CA). FuGENE 6 was purchased from Roche Diagnostics. The kits for agarose purification and cloning were purchased from Qiagen (Valencia, CA). The ceruloplasmin promoter-containing luciferase vector was a kind gift of Dr. Colin Bingle (University of Sheffield, Sheffield, United Kingdom). The clusterin promoter was provided by Dr. Martin Tenniswood (Notre Dame, South Bend, IN). The human glutathione peroxidase...
promoter was generated by PCR using primers designed specifically for the promoter. The resulting PCR product was ligated into a luciferase expression vector, pGL3 basic vector (Promega, Madison, WI), and positive clones were designated as pHGXP.

**Computer- and Literature-Based Search for Potential Ovarian Cancer-Specific Promoters.** We systematically searched the literature including the data of cDNA microarray and the serial analysis of gene expression databases of the National Center for Biotechnology Information to identify genes that are overexpressed in human ovarian cancer. We then determined whether the promoters of these genes were mapped and available.

**PCR.** Each PCR reaction contained 10× PCR buffer (Promega), 1 μl of Taq polymerase (Promega), 10 mM dNTP, 50 ng of template DNA, 100 pmol/μl forward and reverse primers, and distilled water to a final volume of 50 μl. Reactions were performed at 94°C for 120 s, denaturing at 94°C for 60 s, primer annealing at 65°C for 120 s, and elongation at 72°C for 60 s for 5 cycles, followed by 6 min for extension in a DNA thermocycler (T3; Biometra, Hercules, CA). The products were cut out of the gel and prepared for cloning using the QIAprep agarose purification kit (Qiagen).

**Cell Lines and Culture.** The cancer cell lines OVCA 420, 432, ES-2, SKOV3.ip1, MDAH 2774, and CHL1 were grown in 1:1 of Medium 199 and RPMI (Invitrogen). The immortalized ovarian surface epithelial (IOSE) cells were a kind gift of Dr. Nellie Auersperg (University of British Columbia, Vancouver, British Columbia, Canada) and were grown in 1:1 of Medium 199 modified and MCDB 105 trace elements (Sigma-Aldrich, St. Louis, MO). All media were supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 0.2% antimycotic-antibiotic mixture (10,000 units/ml penicillin G, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B; Invitrogen).

**Construction of the Luciferase Reporter Plasmids Containing Mutant Ceruloplasmin Promoters.** Three luciferase expression vectors containing ceruloplasmin deletion mutant promoters were generated and named CERUp-248, CERUp-226, and CERUp-219. The CERUp-248 construct contains the ceruloplasmin promoter region from nucleotide −248 to −15, where −248 to −240 spans a putative AP-1 binding site within the pGL3 basic luciferase expression vector. The CERUp-226 construct contains the ceruloplasmin promoter region from nucleotide −226 to −15, where −226 to −211 spans a progesterone receptor recognition sequence. The CERUp-219 lacks the progesterone receptor recognition sequence and contains nucleotides −219 to −15. The forward and reverse primers used in the PCR reaction to generate each deletion mutant promoter were designed to contain KpnI and BglII restriction enzyme sites, respectively, to facilitate subsequent ligation into a KpnI and BglII-digested pGL3 basic vector. The CERUp-248 ceruloplasmin promoter plasmid was amplified from the forward primer 5′-GCGGATCCCTATTTTTCATGCAGAG-3′ and the reverse primer 5′-AGGTGCGCACTCGAG-3′, the CERUp-226 ceruloplasmin promoter fragment was generated from the forward primer 5′-AGGTCGACACTTTTGGTTGTTTA-3′ and the CERUp-219 was generated from the forward primer 5′-GATCCGAGAATCTTGCAGTTTGGTTA-3′. The nucleotide sequence of the CERUp-219 promoter fragment was confirmed by DNA sequencing performed at the DNA Core Facility at The University of Texas M. D. Anderson Cancer Center confirmed sequences for all positive clones. The plasmids were transformed into One Shot TOP10 Chemically Competent DH5α Escherichia coli cells (Invitrogen), and colonies were screened for successful cloning by purification using the QIAprep Spin Miniprep Kit Protocol (Qiagen) and restriction enzyme digestion.

**Transient Transfection Assays.** Cells were split into 6-well plates at 4 × 10^5 cells/well, and transient transfection experiments were performed 24 h after plating at 60–70% confluence. A total of 2.2 μg of plasmid DNA [2 μg of ceruloplasmin promoter-driven luciferase plasmid (CERUp-487)] or deletion constructs (CERUp-248, CERUp-226, and CERUp-219) and 0.2 μg of pRL-TK internal control plasmid (Promega) were mixed in OPTI-MEM serum-free media and incubated with 4.2 μl of FuGENE for 20 min at room temperature before transfection of the cells. The pRL-TK internal control plasmid was routinely used to check for uniformity of transfection. The cell lysates were harvested after incubation at 37°C for 48 h. For stimulation with 50 nm 1-O-tetradecanoyl phorbol-13-acetate (TPA; Promega), transfected SKOV3.ip1 cells were incubated for 6 h at 37°C with TPA before harvesting the cells. To suppress c-jun transcripational activity, we pretreated these cells for 30 min with 60 μM SP600125 (Tocris Inc., Ellisville, MO), a specific inhibitor of c-jun N-terminal kinase, before TPA stimulation.

**Determination of Luciferase Activity.** After 48 h of transfection, the cells were washed with 1× PBS and harvested in 200 μl of 1× passive lysis buffer provided in the luciferase assay kit (Promega). The samples were subjected to one cycle of freeze-thaw and centrifuged briefly. The supernatant was assayed using the luciferase substrate followed by the Stop-N-Glo Buffer provided by the dual luciferase kit (Promega). The activity was determined in a luminometer (DT20/20; Promega).

**Electrophoresis Mobility Shift Assay.** The nucleotide sequence of the ceruloplasmin AP-1 binding site is 5′-TACCAATTTGAGTTTGAAGAC-3′. Complementary oligonucleotides were denatured, reannealed, and 32P-end labeled using T4 polynucleotide kinase (Promega), and then purified via phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The nuclear lysates were extracted from TPA-treated cells (50 μM) as described previously (21). In binding studies, 10 μg of nuclear extracts were incubated with 5 ng of γ32P-end-labeled probes in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl2, 2.5% glycerol, 0.5 mM EDTA, 0.5 mM DTT, and 1.25 μg of poly(dI-dC),poly(dI-dC) (Amersham Biosciences, Piscataway, NJ). After 15 min at room temperature, the mixture was terminated by the addition of 10× gel loading buffer [250 mM Tris-HCl (pH 7.5) and 40% glycerol] and underwent electrophoresis at 4°C in a 5% nondenaturing polyacrylamide gel (acrylamide:bis-acrylamide, 50:1) with 0.5× Tris-Borate-EDTA and 2.5% glycerol. The gel was dried on Whatman filter paper before exposure to X-ray films. In the competition experiments to determine binding specificity, nuclear extracts were preincubated with a 200-fold excess of cold ceruloplasmin AP-1 site oligo before the addition of 32P-labeled probes. In the supershift assays, 2 μg of c-jun antibody or signal transducer and activator of transcription 3 (STAT3) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were preincubated with nuclear extracts before addition of the 32P-labeled probes.

**Chromatin Immunoprecipitation Assay.** This was performed to determine the in vivo binding of c-jun to the promoter region of ceruloplasmin. SKOV3.ip1 cells were fixed with 1% formaldehyde, washed, and lysed in cell lysis buffer [5 mM HEPES (pH 8.0), 85 mM KCl, and 0.5% NP40] at 4°C for 30 min. After homogenization, the nuclei were then lysed in 100 μl of nuclear lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS]. The lysate was sonicated on ice, and the supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 6.8), and 167 mM NaCl). One μg of c-jun antibody (Santa Cruz Biotechnology) was added to 1 ml of the lysate, and the resulting solution was then pulled down by protein G-conjugated magnetic Dynabeads (Dynal Biotech). The beads were washed with wash buffer [0.1 M sodium phosphate buffer (pH 6.8) and 0.1% Tween-20] four times, and the bound protein was eluted twice with 30 μl of 1 M citrate (pH 3.0). Then, 240 μl of extraction buffer (0.1% SDS, 50 mM NaHCO3, 5 μl of 10 mg/ml RNase A, and 18 μl of 5 M NaCl) were added to the pooled elute and incubated at 65°C overnight. The reverted DNA was purified with a miniprep spin column (Qiagen) and then eluted in 50 μl of 1× Tris-HCl (pH 8.0). The ceruloplasmin promoter region of interest was amplified by primers 5′-CCTAGGCTCCAAAACAACTAC-3′ and 5′-GGAGCTCTAAGAAGAATCTTTCT-3′ using the PCR program: 95°C for 1 min; 72°C for 1 min; followed by 72°C for 1 min. The amplified DNA was cloned into the pGL3 luciferase vector, DNA sequencing confirmed the sequences, and then purified using the QIAprep Spin Miniprep Kit Protocol (Qiagen) and restriction enzyme digestion.

**Immunohistochemistry (IHC) Analysis and Histological Scoring.** All ovarian cancer specimens used in this study were papillary serous, stage IIIC poorly differentiated ovarian carcinomas. The immunoperoxidase staining method was modified from the avidin-biotin complex technique (17). In brief, slides were incubated for 3 h at room temperature with anti-ceruloplasmin antibody (diluted 1:200, Kent Laboratories, Bellingham, WA) and anti-c-jun antibody (diluted 1:100, Santa Cruz Biotechnology). After extensive washings with PBS, the slides were incubated with biotinylated horse antimouse IgG or biotinylated goat antirabbit IgG antibody at 1:200 in PBS. The samples were incubated with avidin-biotin complex-horseradish peroxidase conjugate and developed using aminoethylcarbazole chromogen (Sigma-Aldrich) as a substrate. Positive signals were visualized by light microscopy at high power.
RESULTS

Identification of Potential Ovarian Cancer-Specific Promoters. Our results from the database search revealed several genes that were preferentially expressed in ovarian cancer compared with normal ovarian cells. Specifically, using serial analysis of gene expression library data, the human glutathione peroxidase promoter, clusterin, and ceruloplasmin genes demonstrated an increased expression of 69-, 60-, and 79-fold, respectively, in ovarian cancer cells compared with normal cells (18). When determined with real-time reverse transcription-PCR, ceruloplasmin levels in ovarian cancer were elevated 10,000-fold above normal ovarian cells (18).

Ceruloplasmin Promoter Specificity Is Demonstrable in Ovarian Cancer Cell Lines. To determine whether the ceruloplasmin promoter activity was responsible for the up-regulated ceruloplasmin gene expression in ovarian cancer found in our database search, transient transfection of the CERUp-487 into ovarian cancer cell lines and normal cell lines was performed. The CERUp-487 was significantly activated in five of six ovarian cancer cell lines tested, including OVCA 432, MDAH 2774-c10, ES-2, OVCAR-3, and SKOV3.ip1 (Fig. 1A). In contrast, the ceruloplasmin promoter activity was very low in all four immortalized normal cell lines tested, including IOSE, E6E7 pancreatic cells, Chang liver cells, and Wi-38 lung fibroblast cells. To investigate whether ceruloplasmin promoter activity is specific to ovarian cancer cell lines compared with the standard cytomegalovirus (CMV) promoter, the activity of the ceruloplasmin promoter was compared with the activity of the CMV promoter by normalization of their activity in IOSE cells. When compared with the CMV promoter, the ceruloplasmin promoter demonstrated more specificity in OVCA 420, OVCA 432, ES-2, and SKOV3.ip1 ovarian cancer cell lines (Fig. 1B). Together, these data suggest that the ceruloplasmin promoter activity is specific to ovarian cancer cells compared with immortalized normal cells.

In addition, the clusterin (19) and human glutathione peroxidase (19) promoters were similarly transiently transfected into previously described cell lines, and promoter activity was determined. Despite serial analysis of gene expression analyses showing the expression of both genes to be elevated in ovarian cancers, our data demonstrated minimal activity in all six ovarian cancer cell lines tested and very limited ovarian cancer specificity in either promoter (data not shown).

Ceruloplasmin Overexpression in Ovarian Cancer Tissues. IHC analyses using primary ovarian tumors were performed to establish a positive correlation between the observed increase of ceruloplasmin promoter activity (Fig. 1) and up-regulated ceruloplasmin gene expression. As indicated in Fig. 2, no ceruloplasmin immunoreactivity was noted in the seven normal ovarian tissues tested, whereas eight of 20 (40%) ovarian cancer samples had strong ceruloplasmin reactivity. This is consistent with previous publications that demonstrated ceruloplasmin expression was higher in tumors compared with their normal tissues (12–13).
SKOV3.ip1 cells significantly bound to the ceruloplasmin AP-1 motif chromatin immunoprecipitation assay, and the results of these studies characterized by both electrophoretic mobility shift analysis method and previously.

Representative samples are shown.

 Luciferase activities were then determined and normalized as described in Materials and Methods. The CERUp-248 represents a truncated ceruloplasmin promoter that includes the AP-1 consensus site. CERUp-226 represents the AP-1 site-lacking, progesterone receptor recognition sequence-containing mutant construct. CERUp-219 lacks both the AP-1 and the progesterone receptor recognition sequences. B, TPA activates ceruloplasmin promoter. As confirmatory evidence that the AP-1 site is critical for optimal ceruloplasmin promoter activity, cells were treated with 50 ns TPA for 6 h to stimulate c-jun activity. C, requirement of c-jun activity for the activity of ceruloplasmin promoter. SKOV3.ip1 cells were pretreated with 60 μM SP600125 for 30 min, stimulated with 50 ns TPA for 6 h, and harvested. Luciferase activities were then determined and normalized as described previously.

The functionality of the ceruloplasmin AP-1 site was further characterized by both electrophoretic mobility shift analysis method and chromatin immunoprecipitation assay, and the results of these studies are summarized in Fig. 4. Nuclear extracts isolated from TPA-treated SKOV3.ip1 cells significantly bound to the ceruloplasmin AP-1 motif as indicated by multiple shifted 32P bands in Fig. 4A, Lane 2. The competition experiment showed that cold ceruloplasmin AP-1 site successfully competed out the binding of nuclear extracts to the ceruloplasmin AP-1 site, indicating binding specificity. In the supershift experiments (Fig. 4A), the c-jun-containing nuclear complex, indicated by the arrow, was specifically recognized by the c-jun antibody (Fig. 4A, Lane 4) but not by the STAT3 antibody (Fig. 4A, Lane 5). Consistently, chromatin immunoprecipitation assay demonstrated in vivo binding of c-jun to the ceruloplasmin promoter (Fig. 4B). Together, these binding studies demonstrate that the AP-1 site in the ceruloplasmin promoter is a binding element specifically targeted by the transcription factor c-jun and is critical for optimal activity of the ceruloplasmin promoter.

c-jun Overexpression in Ovarian Cancer Tissues. Because we demonstrated the ceruloplasmin AP-1 site to be responsible for optimal promoter ceruloplasmin activity and the ceruloplasmin AP-1 site to interact with c-jun, we hypothesized that c-jun may also be overexpressed in ovarian cancer and thereby drive the expression of ceruloplasmin. Using IHC analyses in ovarian cancer tissues with an antibody against c-jun, we demonstrated that ovarian cancer tissues stained strongly for nuclear c-jun, whereas normal ovarian tissues did not (Fig. 5). Indeed, 16 of 23 (70%) human ovarian cancer specimens expressed nuclear c-jun, whereas zero of seven normal ovarian tissues stained. Furthermore, a significant and direct correlation ($r = 0.7, P = 0.007$) was found between the levels of ceruloplasmin and c-jun in the 15 ovarian cancer specimens stained for both proteins. These findings suggest that the oncoprotein, c-jun, expressed at high levels in ovarian cancers, may serve as a transcriptional activator of ceruloplasmin in ovarian cancer.

The CERUp-248 Demonstrates Cancer Specificity in Vitro and in Vivo. Because we determined that the CERUp-248 construct demonstrated higher promoter activity than the CERUp-487, we sought to determine whether ovarian cancer specificity was maintained with the truncated CERUp-248 construct. After transient transfection of the CERUp-248 into SKOV3.ip1 cells, transcriptional activity of

Fig. 3. Functional characterization of ceruloplasmin promoter. A, AP-1 site is required for optimal activity of ceruloplasmin promoter. The serially deleted plasmids CERUp-487, CERUp-248, CERUp-226, and CERUp-219 were introduced into SKOV3.ip1 cells, and luciferase activity was determined as described in Materials and Methods. The CERUp-248 represents a truncated ceruloplasmin promoter that includes the AP-1 consensus site. CERUp-226 represents the AP-1 site-lacking, progesterone receptor recognition sequence-containing mutant construct. CERUp-219 lacks both the AP-1 and the progesterone receptor recognition sequences. B, TPA activates ceruloplasmin promoter. As confirmatory evidence that the AP-1 site is critical for optimal ceruloplasmin promoter activity, cells were treated with 50 nM TPA for 6 h to stimulate c-jun activity. C, requirement of c-jun activity for the activity of ceruloplasmin promoter. SKOV3.ip1 cells were pretreated with 60 μM SP600125 for 30 min, stimulated with 50 nM TPA for 6 h, and harvested. Luciferase activities were then determined and normalized as described previously.

The functionality of the ceruloplasmin AP-1 site was further characterized by both electrophoretic mobility shift analysis method and chromatin immunoprecipitation assay, and the results of these studies are summarized in Fig. 4. Nuclear extracts isolated from TPA-treated SKOV3.ip1 cells significantly bound to the ceruloplasmin AP-1 motif as indicated by multiple shifted 32P bands in Fig. 4A, Lane 2. The competition experiment showed that cold ceruloplasmin AP-1 site successfully competed out the binding of nuclear extracts to the ceruloplasmin AP-1 site, indicating binding specificity. In the supershift experiments (Fig. 4A), the c-jun-containing nuclear complex, indicated by the arrow, was specifically recognized by the c-jun antibody (Fig. 4A, Lane 4) but not by the STAT3 antibody (Fig. 4A, Lane 5). Consistently, chromatin immunoprecipitation assay demonstrated in vivo binding of c-jun to the ceruloplasmin promoter (Fig. 4B). Together, these binding studies demonstrate that the AP-1 site in the ceruloplasmin promoter is a binding element specifically targeted by the transcription factor c-jun and is critical for optimal activity of the ceruloplasmin promoter.

c-jun Overexpression in Ovarian Cancer Tissues. Because we demonstrated the ceruloplasmin AP-1 site to be responsible for optimal promoter ceruloplasmin activity and the ceruloplasmin AP-1 site to interact with c-jun, we hypothesized that c-jun may also be overexpressed in ovarian cancer and thereby drive the expression of ceruloplasmin. Using IHC analyses in ovarian cancer tissues with an antibody against c-jun, we demonstrated that ovarian cancer tissues stained strongly for nuclear c-jun, whereas normal ovarian tissues did not (Fig. 5). Indeed, 16 of 23 (70%) human ovarian cancer specimens expressed nuclear c-jun, whereas zero of seven normal ovarian tissues stained. Furthermore, a significant and direct correlation ($r = 0.7, P = 0.007$) was found between the levels of ceruloplasmin and c-jun in the 15 ovarian cancer specimens stained for both proteins. These findings suggest that the oncoprotein, c-jun, expressed at high levels in ovarian cancers, may serve as a transcriptional activator of ceruloplasmin in ovarian cancer.

The CERUp-248 Demonstrates Cancer Specificity in Vitro and in Vivo. Because we determined that the CERUp-248 construct demonstrated higher promoter activity than the CERUp-487, we sought to determine whether ovarian cancer specificity was maintained with the truncated CERUp-248 construct. After transient transfection of the CERUp-248 into SKOV3.ip1 cells, transcriptional activity of

Fig. 4. Specific binding of c-jun to the ceruloplasmin promoter. A, specific in vitro binding of c-jun transcription factor to the ceruloplasmin AP-1 site via electrophoretic mobility shift analysis. Five ng of γ-32P-end-labeled ceruloplasmin AP-1 motif were used in all reactions. Ten μg of nuclear extracts were included in all reactions except Lane 1, which contained only c-jun antibody. In the competition experiment (Lane 3), 200-fold excess of cold ceruloplasmin AP-1 motif was preincubated with the nuclear extracts before the addition of the32P-ceruloplasmin AP-1 probe. For the supershift reactions, antibodies specific for c-jun (Lane 4) or STAT3 (Lane 5) replaced the cold ceruloplasmin AP-1 motif (Lane 4). B, in vivo binding of c-jun to the promoter region of ceruloplasmin gene. SKOV3.ip1 cells were fixed, lysed, homogenized, and subjected to chromatin immunoprecipitation analysis for in vivo binding of c-jun to the ceruloplasmin promoter. Before immunoprecipitation, an aliquot of lysate was saved as total input (Lane 1) and used as the positive control for the PCR reactions. For immunoprecipitation, rabbit IgG (Lane 2) was used as the negative control, and c-jun antibody was used for pulling down c-jun-interacting chromatin (Lane 3).
CERUp-248 was determined using the luciferase gene as a reporter. The results of promoter specificity of CERUp-248 are summarized in Fig. 6A. In five of six ovarian cancer cell lines tested, the specificity of the CERUp-248 was demonstrably higher than the IOSE, E6E7, Chang liver, and Wi-38 fibroblast cell lines. Therefore, the truncated CERUp-248 promoter maintains specificity within the ovarian cancer cell lines tested. Taken together with the previous finding that the CERUp-248 promoter activity is higher than the CERUp-487 promoter (Fig. 3A), the CERUp-248 demonstrates more potential for use in gene therapy because of its specificity to cancer cells and its higher activity than CERUp-487.

We additionally examined whether CERUp-248 demonstrates tumor specificity in vivo. Using nude mice bearing SKOV3.ip1 xenografts, CERUp-248 displayed 3.1–7.0-fold higher activities in the tumors compared with those in the normal tissues, including, spleen, liver, kidney, lung, and heart (Fig. 6B). In particular, the activities of CERUp-248 were very limited in livers and lungs: 14.4% and 20%, respectively, of the activity found in tumors. Together, ceruloplasmin promoter demonstrates high tumor specificity both in vitro and in vivo, indicating its potential use in gene therapy.

**DISCUSSION**

In this study, we provide evidence that defines the ceruloplasmin promoter as a CSP. It demonstrates significantly higher transcriptional activity in ovarian cancer cell lines and xenografts compared with immortalized normal cell lines and normal organs, respectively. The difference in the one ovarian cancer cell line that only demonstrated moderate transcriptional activation may be due to the natural biology of ovarian cancer, where ceruloplasmin promoter function may be absent or altered in this specific cell line. Notably, the levels of promoter activity were less in the normal liver, lung, pancreatic, and ovarian cell lines. Therefore, the ceruloplasmin promoter has potential for utilization in the targeted expression of therapeutic genes.

If the ceruloplasmin promoter is applied to gene therapy for ovarian cancer, it would be important to know what percentage of ovarian cancer tissue specimens overexpressed ceruloplasmin because these tissues would likely demonstrate high transcriptional levels of the ceruloplasmin promoter. In our study, IHC studies performed in high-grade ovarian cancer tissues revealed 40% ceruloplasmin overexpression compared with normal cells. These results suggest that if the ceruloplasmin promoter were applied to a gene therapy setting, then this therapy would potentially target 40% of high-grade ovarian serous carcinomas presuming that all 40% of positively staining tumors are derived from transcriptional up-regulation. This estimate may even be within the lower limits of expression as we have shown that six of seven ovarian cancer cell lines show transcriptional activity of the ceruloplasmin promoter.

We determined that the AP-1 binding site was a regulatory element contained within the ceruloplasmin promoter and was required for optimal ceruloplasmin promoter activity. Analysis of the sequences between nucleotides −487 and −248 revealed that the only known transcriptional factor was the AP-1 binding site. Therefore, the increased transcriptional activity seen in the CERUp-248 compared with CERUp-487 was the result of the AP-1 binding site and possibly a negative regulatory component in the CERUp-487 not yet identified. Importantly, the interacting element of the AP-1 site of the ceruloplasmin promoter was found to be targeted directly by the transcriptional factor c-jun. Because the activity of the ceruloplasmin promoter was specifically enhanced in ovarian cancer, we hypothesized that this
selective expression was activated by nuclear c-jun levels. Indeed, we show that levels of nuclear c-jun positively and significantly correlate with those of ceruloplasmin in ovarian tumor specimens. We treated the transfected SKOV3.ip1 cells with TPA, which is known to be a potent activator of AP-1. In the setting of stimulation with TPA, the AP-1 binding site has also been referred to as the TPA-response element (22). TPA stimulation was used as confirmatory evidence that the AP-1 binding site of the ceruloplasmin promoter responded to activation. In addition, we found that inhibition of c-jun transcriptional activity, using SP600125, resulted a great reduction of ceruloplasmin promoter activity, further indicating the requirement of c-jun for ceruloplasmin promoter function.

In conclusion, we found that the ceruloplasmin promoter demonstrates high activity in ovarian cancer and may have utility in driving the expression of therapeutic genes. Because the expression of ceruloplasmin has been shown to be elevated in other cancer types (12, 13, 19, 20), our findings implicate a potential use of ceruloplasmin in other cancers. Furthermore, we identified the AP-1 regulatory element within the promoter that is required for optimal promoter activity. These regulatory elements could be exploited and used as enhancer elements to the promoter, thereby increasing the efficacy of a therapeutic gene. Together, findings reported in this study provide support for the use of ceruloplasmin promoter as a potentially new treatment strategy in ovarian cancer.

ACKNOWLEDGMENTS

We thank Dr. Colin Bingle for the original ceruloplasmin promoter construct, Dr. Nellie Auerberg for IOSE cells, and Dr. Karen Lu for the ES-2 ovarian cancer cells. We thank Dr. Martin Tenniswood for the clustatin promoter.

REFERENCES

Selective Activation of Ceruloplasmin Promoter in Ovarian Tumors: Potential Use for Gene Therapy

Christine M. Lee, Hui-Wen Lo, Ru-Ping Shao, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/5/1788

Cited articles
This article cites 20 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/5/1788.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/64/5/1788.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.